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# An isothermal titration calorimetry study of phytate binding to lysozyme

## A multisite electrostatic binding reaction

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**Abstract** Isothermal titration calorimetry (ITC) was used to detect phytate binding to the protein lysozyme. This binding interaction was driven by electrostatic interaction between the positively charged protein and negatively charged phytate. When two phytate molecules bind to the protein, the charge on the protein is neutralised and no further binding occurs. The stoichiometry of binding provided evidence of phytate–lysozyme complex formation that was temperature dependent, being most extensive at lower temperatures. The initial stage of phytate binding to lysozyme was less exothermic than later injections and had a stoichiometry of 0.5 at 313 K, which was interpreted as phytate crosslinking two lysozyme molecules with corresponding water displacement. ITC could make a valuable in vitro assay to understanding binding interactions and complex formation that normally occur in the stomach of monogastric animals and the relevance of drinking water temperature on the extent of phytate–protein interaction. Interpretation of ITC data in terms of cooperativity is also discussed.

**Keywords** Allostery · Cooperative binding · ITC · Phytase · Digestion

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## Introduction

Phytic acid is a *myo*-inositol with six orthophosphate groups linked to it by phosphoester bonds. The dissociated form of the acid, which exists above a pH of 2, named phytate, is known to bind to a number of trace metals, colloidal clays and proteins [1–5]. These studies suggest that a phytate ion can bind to positively charged proteins and minerals since it occurs as a set of negatively charged species whose charge composition becomes more negative with increasing pH [1, 6, 7]. Seeds including cereals, legumes and nuts all contain phytate with concentrations ranging from 5 to 50 g per kg [8]. This presents a nutritional problem, as phytate can chelate ions with biological functions including zinc and iron [2, 9]. It has also been suggested that phytate could form non-digestible complexes with exogenous protein sources and endogenous proteins involved in digestion, causing a reduction in growth in humans [10, 11]. The anti-nutritional effects of phytate are not limited to the human population. Nutritional efficiency is important in the farming industry. Pigs and poultry are negatively affected in terms of growth performance due to phytate present in feed [12, 13]. This has led to supplementation with phytase to animal feed. Phytase is an enzyme that hydrolyses the phosphate esters present on phytate [14]. The global phytase market was evaluated to be worth $350 million annually and accounts for more than 60 % of the total feed enzyme market in 2010 [15]. This year, the global feed enzyme business is now worth close to $1 billion per year, with the phytase market being in the region of $550–600 million.

Although a number of molecular mechanisms have been put forward to explain the anti-nutritional effects of phytate, there has been little thermodynamic characterisation of them. The exceptions are the determination of the

association constants between phytate and ions like zinc, and the thermal stability of proteins in the presence of phytate [1, 2]. Without proper thermodynamic characterisation, the strength and specificity of phytate–protein interactions can only be estimated. It has been suggested that the anti-nutritional effects of phytate on pig and poultry nutrition are a multifaceted subject that requires an understanding of how phytate interacts with minerals, proteins and enzymes including phytase in vivo and in vitro [16]. Isothermal titration calorimetry (ITC) is a technique that provides enthalpy ($\Delta H$), entropy ($T\Delta S$) and Gibbs free energy values ($\Delta G$) of binding by measuring the change in heat between two interacting partners in solution. It has been used successfully to characterise a diverse range of binding interactions [17], including how the β-propeller phytase hydrolyses insoluble calcium phytate salts and prevents phytate from binding to metal ions [18]. More recently, ITC has been used to determine how phytate could interact with amines in the cellular medium to gain a greater understanding of its function within cells [19]. However, it has not been used to measure the binding energetics between positively charged proteins and phytate.

In this study, we have used lysozyme as a model protein as it is known to interact with phytate. Lysozyme is positively charged under the conditions used in this ITC study, whilst phytate is negatively charged. Consequently, this is a good model for an electrostatic binding interaction between a protein and ligand. In addition to this, digestive enzymes such as trypsin are also positively charged, meaning that this study could shed light on the mechanism of the anti-nutritional effect of phytate.

## Materials and methods

Hen egg-white lysozyme, potassium phytate (≥95 % purity), acetic acid (99.8 %) and ammonium acetate were all sourced from Sigma-Aldrich, Gillingham, U.K. Milli-Q water from a Merk Millipore (Darmstadt, Germany) fountain was used to prepare the reagents, and a Puradisk™ 0.45-μm filter (GE Healthcare, Buckinghamshire, UK) was used in the aggregation studies. A FiveEasy pH meter (Mettler-Toledo International, Ohio, US) was used to measure the pH of the solutions. A Jenway spectrophotometer (Bibby Scientific Limited, Staffordshire, UK) was used to measure the absorbance of the solutions.

### Isothermal titration microcalorimetry

A Nano-ITC instrument (TA instruments, Utah, US) was used to measure the binding thermodynamics between phytate and lysozyme at 283, 298 and 313 K. Phytate was dissolved in 10 mM ammonium acetate buffer at pH 4. The lysozyme was dialysed overnight in a 10 mM ammonium acetate buffer at pH 4. All solutions were degassed prior to the titrations. Six mM phytate was loaded into the syringe and titrated into the sample cell, which contained 400 μM lysozyme. At least 23 × 1.5 μL 6 mM phytate injections into 400 μM lysozyme were made over the course of the titration. In each titration, the reference cell contained water. The time delay between each injection was variable to ensure the baseline returned to normal, and the RPM (rotations per minute) was set to 200 to ensure thorough mixing of the cell. Data analysis was performed using the NanoAnalyze software (TA instruments, Utah, USA). The enthalpy of dilution of phytate was subtracted from the raw data. Raw heat data were integrated peak by peak and normalised per mole of injectant to get a plot of observed enthalpy change per mole of phytate against phytate–protein molar ratio. The first injection was erroneous due to diffusion effects and although included in the data, should be disregarded. Calibration was performed by 2 % propanol dilution [20].

### Nanoparticle tracking analysis (NTA)

NTA (NanoSight Ltd., Wiltshire, UK) can visualise nanoparticles in a liquid suspension. NTA has 3 basic stages: (1) video capture, (2) setting of analysis parameters and (3) tracking analysis. The NanoSight (LM14) was used in this experiment. The particles scatter light which is visualised using an optical microscope. A video is then captured to record the tracks of the particles. Two controls were used in this study. Solutions of 6 mM phytate and 400 μM lysozyme were analysed separately using NTA to determine the amount of particles. Before injecting the protein solution into the port, the solution was filtered to remove particles larger than 0.45 μM using a Puradisk™ filter. To determine the amount of crosslinking between phytate and lysozyme, the solutions were thoroughly mixed and 1 mL of the solution was injected into the cell. Temperature was held constant at 298 K during the experiment, using the temperature control on the LM14.

## Results and discussion

Titration of phytate into 10 mM ammonium acetate buffer, pH 4.0, showed a negligible $\Delta H$ (see Supplementary Fig. 2). The binding isotherms of all three titrations of phytate into lysozyme in a 10 mM ammonium acetate buffer, pH 4.0, at 283, 298 and 313 K had a clear measurable change in enthalpy ($\Delta H$) and showed evidence of two stages for the interaction. Note, ammonium acetate was chosen due to the small change in enthalpy of ionization for this buffer [21]. The first stage showed

increasingly exothermic $\Delta H$ with each injection that produced a downward curvature in the binding isotherm below 1 phytate–lysozyme molar ratios (Fig. 1, Supplementary Figs. 1 a–c). The second stage was comprised of a plateau in the exothermicity followed by a decline in exothermic $\Delta H$ with each injection associated with saturation of binding sites. The curves for all three titrations had an exothermic peak followed by a shallower endothermic peak in the initial injections (Fig. 1, Supplementary Figs. 1 a–c). This is observed as a sharp peak followed by a dip in raw heat rate that slowly equilibrates. The dip disappears by the 3rd injection at 283 K, the 4th injection at 298 K and the 6th injection at 313 K. This was most pronounced at 313 K (Fig. 1). This is evidence of a fast exothermic reaction and a slower endothermic reaction taking place during binding in the initial injections. The presence of overlapping exothermic and endothermic reactions would explain why the first stage of the binding isotherm has lower calculated exothermic values for the initial injections. The fast exothermic event is evidently binding of the phytate to the lysozyme. The authors' hypothesis based on these data is that the slower endothermic reaction was related to crosslinking of the lysozyme molecules by the phytate. Similar mixed exothermic and endothermic reactions are observed when epigallocatechin gallate is titrated into proline-rich proteins [22]. To detect aggregates of lysozyme caused by crosslinking at low phytate–lysozyme ratios, nanoparticle tracking analysis (NTA) was used (Fig. 2). NTA was able to detect complex formation at very low phytate concentrations which mimicked the conditions during the initial injections in the ITC. The authors contend that the slow endothermic reaction is the crosslinking of lysozyme by phytate, and the reason that this is endothermic is due to the displacement of the water from the lysozyme surfaces as they are brought together by phytate (Fig. 3). Close observation suggests that the shape of the initial stage of the binding isotherm is different at different temperatures (Fig. 4a–c) and that this is reproducible (Supplementary Figs. 3 a–c). At 283 and 298 K, the endothermic contribution reduces with each injection. At 313 K, the endothermic contribution is a sigmoidal shape with an apparent stoichiometry of 0.5 phytate per lysozyme, which agrees with two lysozymes being linked together.

The second phase of the titration binding isotherm (after 1 mol ratio) can be fitted to a one-site binding model using an n value greater than one (Fig. 1, Supplementary Figs. 1 a–c). The experimentally derived binding isotherm data for the second stage of the titration fit the one-site binding model reasonably accurately (Fig. 1, Supplementary Figs. 1 a–c). The calculated thermodynamic values estimated by the one-site model have to be treated with caution. The one-site binding model assumes that the $\Delta G$ and $\Delta H$ values for the first and second binding interaction are identical. The thermodynamic constants were calculated for repeated ITC titrations. The mean value and standard deviation presented for $\Delta H$, $T\Delta S$ and $\Delta G$ at 283, 298 and 313 K are shown in Table 1. The change in Gibbs free energy ($\Delta G$) of binding became more favourable with increasing temperature. The change in $\Delta G$ was not as large

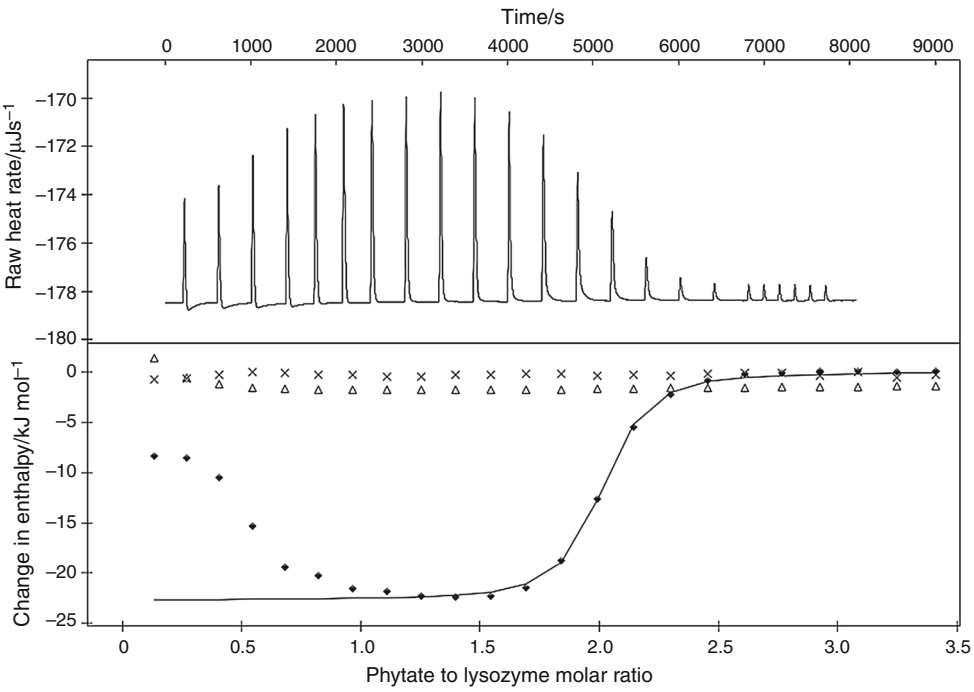


**Fig. 1** Isothermal titration calorimetry (ITC) of phytate (6 mM) into lysozyme (0.4 mM) in 10 mM ammonium acetate buffer, pH 4, at 313 K showing the experimentally derived curve with raw heat rate/μ $Js^{-1}$ versus time/s (*top*) and the calculated binding isotherm with change in enthalpy/kJ $mol^{-1}$ versus phytate–lysozyme molar ratio (*bottom*)

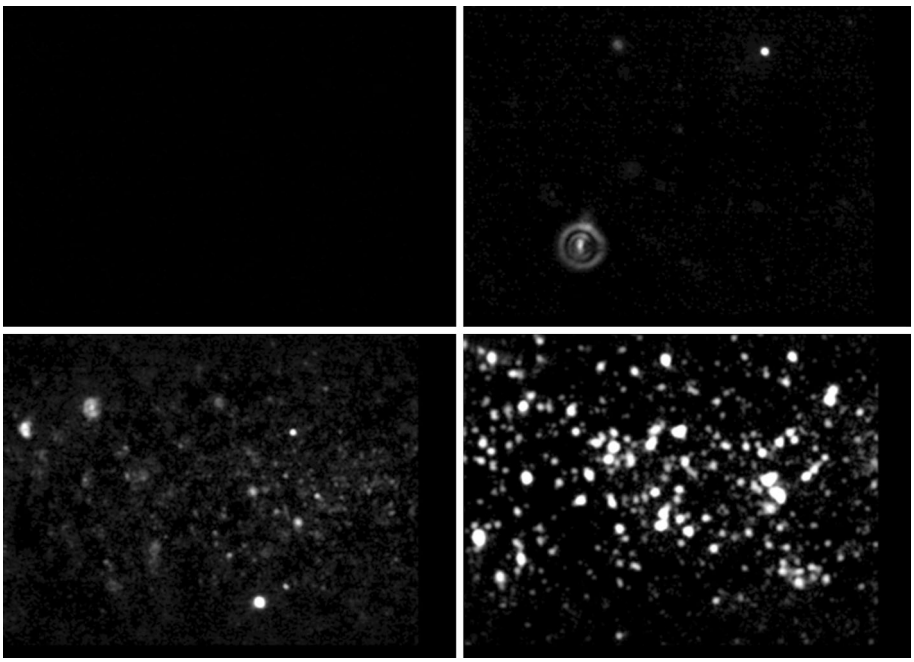

**Fig. 2** Microscopy images obtained during the nanoparticle tracking analysis of phytate, buffer and lysozyme mixtures. *Top right*: negative control; 6 mM phytate in 10 mM ammonium acetate buffer. *Top left*: negative control; 400 μM lysozyme in 10 mM ammonium acetate buffer at pH 4. *Bottom left*: test sample; 6 mM phytate with 400 μM lysozyme (at a molar ratio of 0.132) in 10 mM ammonium acetate. *Bottom right*: 6 mM phytate with 400 μM lysozyme (at a molar ratio of 0.297) in 10 mM ammonium acetate. All samples at pH 4

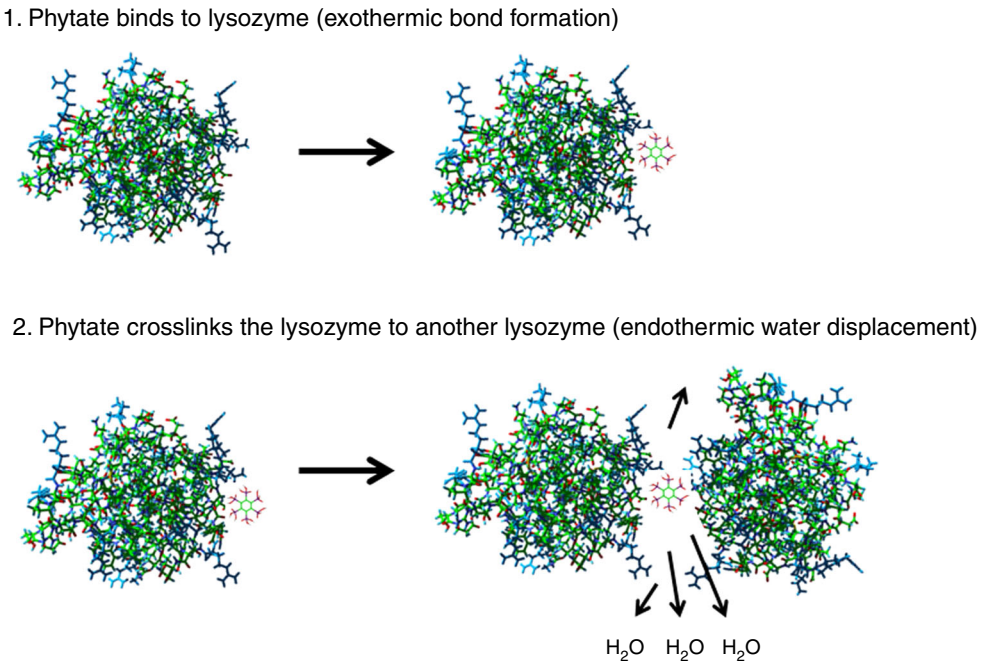


**Fig. 3** Proposed binding events during initial ITC injections of phytate into a lysozyme solution

**Table 1** Thermodynamic values derived for phytate–lysozyme interaction in 10 mM ammonium acetate buffer at pH 4 using the one binding site model on the second stage of the binding isotherm

| Temperature/K | $\Delta H$/kJ mol$^{-1}$ | $T\Delta S$/kJ mol$^{-1}$ | $\Delta G$/kJ mol$^{-1}$ | Stoichiometry/n |
|---|---|---|---|---|
| 283 | −31.6 ± 1.2 | −2.2 ± 1.9 | −29.5 ± 0.8 | 1.63 ± 0.07 |
| 298 | −25.0 ± 0.9 | 8.0 ± 1.0 | −33.0 ± 0.5 | 1.91 ± 0.06 |
| 313 | −22.6 ± 0.3 | 12.4 ± 0.7 | −34.9 ± 0.4 | 2.01 ± 0.09 |

The standard deviations are also included

as the change in $\Delta H$ due to the compensating change in the calculated $T\Delta S$. The stoichiometry also changed with temperature with an n of $1.63 \pm 0.07$ at 283 K, $1.91 \pm 0.06$ at 298 K and $2.01 \pm 0.09$ at 313 K. Interpretation of the second stage of the titration is that binding of the phytate to the lysozyme is the dominant feature providing a simple exothermic titration curve. Further aggregation of lysozyme is likely to be occurring and is observed as a slow exothermic reaction in the 13–15th injection in the curve at 298 and 313 K (that can be observed as a shoulder after the main injection peak). The change in stoichiometry as the temperature rises suggests that the lysozyme–phytate complexes differ at different temperatures. At low temperatures, less phytate can bind to the lysozyme, presumably due to extensive crosslinking, and at higher temperatures, more phytate can bind due to less extensive crosslinking. As the temperature increased, the value of the $\Delta H$ became less exothermic, suggesting a positive change in heat capacity ($\Delta C_p$). $\Delta C_p$ provides thermodynamic information regarding the change in hydration upon protein–ligand binding [23, 24]. In most ITC studies, a negative $\Delta C_p$ is interpreted as an indication that water is being displaced from apolar groups during hydrophobic interaction [25]. A positive $\Delta C_p$ is consistent with binding of a hydrophilic molecule like phytate.

The contribution from $T\Delta S$ during binding under aqueous conditions is difficult to interpret. It has been suggested in ITC studies of tannins binding to proteins that $T\Delta S$ has a systematic error, as any endothermic interaction subtracts from the exothermic heat changes detected by the ITC [26]. It has also been suggested that $T\Delta S$ is not a measure of how ordered the system is and it should be taken as a measure of energy dispersal [27]. When phytate binds to lysozyme, there will presumably be some change in the degrees of freedom in comparison with the non-bound state where the binding partners exist separately. Additionally, upon binding, both water and buffer ions will be displaced. All of these will contribute either favourably or unfavourably to the $T\Delta S$ value.

It is assumed that the interaction between negatively charged phytate phosphate groups and positively charged amino acids is electrostatic. To confirm that the interaction is predominantly electrostatic, the concentration of ammonium acetate was increased from 10 to 300 mM (Fig. 5). The amount of heat detected was reduced significantly in 300 mM salt, indicating that the interaction is purely electrostatic as high salt concentrations have screened any electrostatic interactions. Furthermore, this indicates that hydrogen bonding between the phosphate groups on the phytate and amino acids on the protein is unlikely to happen. To understand the binding interaction in its entirety, the sequence of binding events and the charge of molecules have to be considered. Lysozyme has

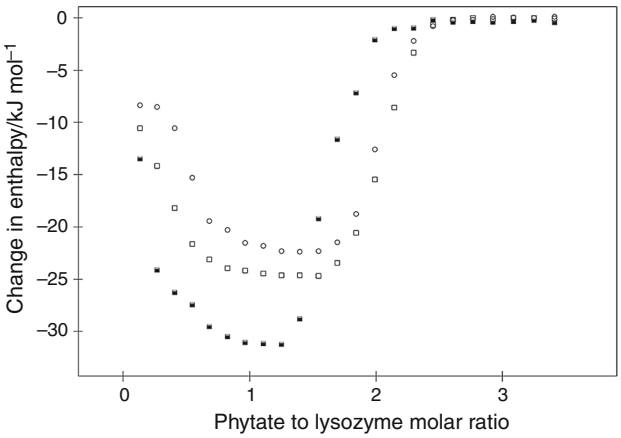


**Fig. 4** Binding isotherm from the titrations of phytate (6 mM) into lysozyme (0.4 mM) in 10 mM ammonium acetate, pH 4, at 283 K (*closed square*), 298 K (*open square*) and 313 K (*open circle*)

a positive charge at pH 4 of +12 [28], and phytate has a negative charge of −6 [5]. Binding of the first phytate would result in a decrease in the net positive charge on the lysozyme from +12 to +6. In the next binding event, the net positive charge on the protein diminishes from +6 to 0. It is possible that the lack of positive charge on the lysozyme then allows further lysozyme aggregation as the charge repulsion is diminished. The phytate–lysozyme aggregates could sterically hinder access of other phytates to lysozyme, and they could also produce a shield of negative charge around the protein aggregate surface thereby repelling unbound phytate molecules. Increasing the temperature would break down the weakly associated aggregates into smaller complexes which would increase the stoichiometry by exposing additional binding sites. Although the conditions in this ITC study are not physiological, it is interesting to note that the interaction between phytate and lysozyme still occurred at 100 mM ammonium acetate concentration at pH 4.0, which is approximately the same ionic strength and pH of the human stomach after a meal [29, 30] (Fig. 5).

The non-sigmoidal curve associated with binding of a molecule to different sites has often been explained as being evidence of allostery or cooperativity [31]. These terms are commonly applied to multiple binding site interactions and could be applied to phytate binding to lysozyme. As allostery and cooperativity are terms readily applied to ITC data that do not have a sigmoidal shape, this will be discussed in more detail and the phytate binding to lysozyme discussed in terms of this background.

The term cooperativity has been applied to interactions where binding to the first site either energetically favours subsequent binding to another site (positive cooperativity) or makes subsequent binding less favourable (negative cooperativity). There are several different binding

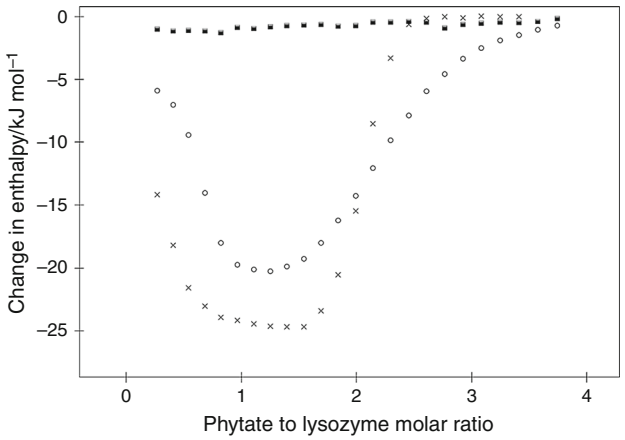


**Fig. 5** Binding isotherm from the titrations of phytate (6 mM) into lysozyme (0.4 mM) in 10 mM (*crosses*), 100 mM (*open circles*) and 300 mM (*black squares*) ammonium acetate buffer pH 4 at 298 K

phenomena where the term cooperativity has been applied, including structural change, complex formation by the titrant, negative electrostatic cooperativity and the nonlinear additivity of the titrant as it is polymerised [32–34]. The term allostery is closely related to cooperativity but is applied to proteins (and protein complexes) where the regulation of binding to one site is modulated by binding to another and is often associated with structural change in the protein. Protein allostery has remained a focal point of biochemistry for the past 50 years due to the role it plays in regulating metabolism. If ITC was able to contribute to this field, it would be an asset. ITC has been used to study both homotropic (the ligand binds to the binding site and changes the affinity of another binding site for the same ligand) and heterotropic (a ligand affects the affinity of another binding site for a different ligand) modulation of protein allostery [35, 36]. However, for homotropic binding, there has been some difficulty in differentiating between binding isotherms that display positive cooperativity and those that demonstrate that a ligand binds to different sites on a protein independently but with different $\Delta H$ values. This has led to the development of simulations to model binding isotherms that attempt to make this differentiation between independent and cooperative binding [31, 37]. The issue arises due to the fact that a protein exhibiting two or more binding sites with similar affinities for the same ligand but different enthalpies produces binding isotherms that are comparable to those previously identified as homotropic positive cooperativity.

Most previous studies claiming to have discovered positive cooperativity in various binding systems using ITC have used the presence of a biphasic isotherm with increasingly favourable negative $\Delta H$ at low molar ratios as proof [38–40]. A favourable increase in negative $\Delta H$ does not necessarily correspond to a favourable increase in negative $\Delta G$ as there is often an opposing $T\Delta S$. In cases where positive cooperativity is suggested, there should be an increase in affinity for the second binding event (increase in negative $\Delta G$) which cannot be seen in the data (Table 1). Here we suggest that positive cooperativity is not required to explain the ITC data for phytate binding to lysozyme. The first stage can be explained by a crosslinking process. If cooperativity was occurring, we would assume it was negative due to the reduction in positive charge on the protein as phytate binds. The authors of this study contend that the use of cooperativity as an explanation for non-sigmoidal binding ITC isotherms can be simplistic and often misleading, especially where complex formation is observed.

To the authors' knowledge, this is the first paper to show that phytate–protein interaction and complex formation are temperature dependent. This has considerable implications for animal nutrition. The core body temperature of pigs and poultry is in the range of 310–340 K, and based on the binding stoichiometry presented herein, this may promote phytate–protein interactions. However, putatively, phytate–protein interactions are more likely to occur in the gastric region of the intestine where pH is more commonly below the isoelectric point of dietary protein [41], and in this context, the relevance of drinking water temperature should be considered. There have been several studies that explored the effect of drinking water temperature on broiler chicken performance, but most are associated with alleviation of stress at high ambient temperature. It has been noted that the performance of broiler chickens raised in high ambient temperatures improved when drinking water temperature was reduced from 316 to 283 K [42]. These effects may be age dependent and/or interact with brooding temperatures [43]. However, the focus of research on drinking water temperature has been restricted to interactions with environmental temperature and heat stress alleviation. The effect of drinking water temperature has not been systematically assessed under conventional terms of reference or specifically examined effects of phytate, phosphorus digestibility or the anti-nutritional effects of phytate. The anecdotal interactions between phytase efficacy and dietary NaCl or $NaHCO_3$ during winter/summer feeding systems and geographical variation in phytase 'super-dosing' effects may be associated with drinking water temperature and its effect on the extent of phytate–protein complex formation. The effect of drinking water temperature on phytase efficacy and the anti-nutritional effect of phytate is an area for further study, and the implications of these interactions for the nutrition of warm- and cold-water aquaculture should be considered.

This study indicates that ITC can be applied to study the binding of phytate to positively charged proteins such as digestive proteins and protein substrates present in the

animal diet. Therefore, it could provide a useful tool to understand the thermodynamics of the anti-nutritional effects of phytate as long as care is taken with the interpretation of the data. As shown here, the stoichiometry can provide information regarding complex formation and the extent of phytate binding. By modifying parameters like pH and the small molecule composition of the aqueous solution, conditions can be made to replicate digesta. Consequently, ITC could be used as a tool to validate current theories regarding the nutritional role of phytate in a controlled and systematic manner and potentially assist in diet formulation.



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